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13. ABSTRACT *(Maximum 200)*

A characteristic feature of human malignancy is the loss of normal cell cycle control. One mechanism by which proteins control the cell cycle is by ubiquitin-mediated destruction of cell cycle regulators. *CDC34*, a gene essential for mitotic cell division in yeast encodes an enzyme required for degradation of yeast cell cycle regulators. The goal of this project is to understand the role of human Cdc34 in cell cycle transition in normal and malignant mammary cells. We hypothesize that human Cdc34 is a cell cycle regulatory protein which modulates the stability of specific cellular proteins by targeting them for degradation. The targets of mammalian Cdc34 are not presently known. We have used a genetic assay called two-hybrid cloning to identify proteins that interact with mammalian Cdc34, screening 1.5 million human cDNAs. 30 cDNA clones found to be active in this assay have been isolated and are currently being analyzed. Surprisingly, four of these clones are known to be involved in the control of meiosis and spermatogenesis, one also has a distinct role in DNA double strand break repair. Thus, it appears from our studies that human Cdc34 may have a novel role in meiosis, recombination, and response to DNA damage in higher organisms by specifically targeting these regulators for ubiquitination. Further studies are currently in progress to characterize the Cdc34-mediated regulation of these interactors and their physiological role in the cell cycle progression and development of breast cancer.

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FOREWORD

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Introduction

Loss of cellular growth control and derangement of normal cell cycle regulation are hallmarks of human malignancy. A fundamental mechanism by which proteins control the mitotic cell cycle is by ubiquitin-mediated destruction of either positive or negative cell cycle regulators such as cyclins and CKIs (cyclin-dependent kinase inhibitors), respectively. *CDC34,* a gene essential for the early phases of the cell division cycle in yeast, encodes a ubiquitin ligase, an enzyme required for ubiquitination and proteolytic degradation of yeast cell cycle regulators (for review see 1). Budding yeast (*S. cerevisiae*) cells mutant in *cdc34* arrest in G1 and are unable to begin DNA replication (2, 3). Isolation and sequencing of the *S. cerevisiae CDC34* gene, which encodes a 295 amino acid protein (2), revealed amino acid similarities to the ubiquitin-conjugating enzyme (E2) family of proteins including the DNA repair gene *RAD6* (4,5), and was named UBC3 (6, for review see 7). The Cdc34 protein consists of a highly conserved catalytic domain common to all UBC enzymes and an unique carboxy terminal extension or tail (2), which is essential for cell cycle function (8, 9). Possible functions of the tail include directing specific association with other regulatory proteins and Cdc34 self association (10, 11).

Highly related functional *CDC34* genes in the human andthe mouse have been cloned and characterized for the first time in our laboratory (12, 13, Pati, D. and Plon, S.E., unpublished) and found to share significant homology at the protein level with *S. cerevisiae.* These studies have shown that human *CDC34* fully complements $cdc34^{ts}$ mutant yeast for growth at the restrictive temperature (12), and also rescues the lethality of the *cdc34* null strain (Plon, S.E., unpublished). Consistent with yeast Cdc34, the human Cdc34 protein is found to be phosphorylated and autoubiquitinated *in vitro* (M. Gobel and S. Plon, personal communication). These results suggest a conserved role for Cdc34 in the cell cycle control of the G1/S transition.

Ubiquitin-mediated proteolysis (also known as ubiquitination) has been implicated in a wide range of cellular processes including cell cycle control, sporulation, and radiation resistance (2, 4, 5, for review see 1). It is a highly conserved process among diverse eukaryotes, where ubiquitin, a small polypeptide of 76 amino acids, is covalently attached to the target protein. This usually directs the protein into rapid proteolysis (for review see 7). Ubiquitin forms a thiolester bond with the ubiquitin-conjugating enzyme (E2). This enzyme alone or in conjunction with a third enzyme E3 (ubiquitin-protein ligase), catalyzes isopeptide bond formation between ubiquitin and the substrate. Ubiquitination of regulatory molecules, including the mitotic B-type cyclins and components that connect sister chromatids (14), play a crucial role in cell cycle progression at several points in the cycle including progress past anaphase (15, 16). It is likely that transformation results in the improper regulation of these proteolytic pathways, but as yet it is not clear whether such regulation is at the level of ubiquitin ligase or the substrates themselves (17). In this context, ubiquitin-dependent proteolysis has recently been shown to be very important in the proper regulation of initiation of DNA replication in yeast (18). In view of the conserved nature of the cell cycle regulation in eukaryotes, it is likely that mammalian cell cycle control is also dependent on ubiquitin-mediated proteolysis and that these events are deregulated in oncogenesis.

Targets of Cdc34 which must be degraded for entry in S phase to occur have been sought for several years in yeast. It is now known that the Cdc34 ubiquitin ligase of *S. cerevisiae* appears to target both G1 cyclins (Clns) and Cdk inhibitors in regulating entry into S phase (for a review see 1). Targets of Cdc34 in budding yeast include a number of cell cycle regulatory molecules including, Cdk inhibitor Sic1, DNA replication protein Cdc6, Cln2/Cdc28 inhibitor Far1, Cln2 itself, and transcription factor GCN4 (19, 20, 21). However, very little is known about the probable targets of Cdc34 in higher organisms, including humans.

The goal of this project has been to understand the role of human Cdc34 in cell cycle transition in normal and malignant mammary cells. We have hypothesized that Cdc34 is a cell cycle regulatory protein in mammalian cells which modulates the stability of specific cellular proteins by targeting them for degradation. The physiological targets of mammalian Cdc34 are not presently known. We have used a yeast-based *in vivo* genetic assay called two-hybrid cloning to identify proteins that interact with mammalian Cdc34. cDNAs found to be active in this assay have been isolated and are currently being analyzed.

The following is a progress report of our first technical objective i.e. the **Identification of Cdc34 Target Proteins.** As described in the original proposal, task #1-5 (see appendix1) were performed over the first year of the grant period (see below for details) and Task # 6 is currently underway.

Body of the report

Methods and Results:

Reagents: Reagents used in the following two-hybrid screening include the Gal4-activation domain (AD) library, the Gal4-DNA binding (DB) vector (pPC97), the yeast host strain MV103 (Mat **a**, *leu2*, *trp1*, *his3*, Gal1:HIS3, Gal1:LacZ, Spal:URA3), and 5 constructs in MV103 for use as reference controls during screening (22, 23). Control plasmids include 1) DB-pPC97+AD-pPC86, 2) DB-pRb+AD-E2F1, 3) DB-Fos+AD-Jun, 4) Gal4+AD, 5) DB-dDP-1+AD-dE2F.

Construction of the DNA Binding-Cdc34 Fusion ("bait"): The full length human *CDC34* cDNA (12) has been cloned into pPC97 (Leu+) *GAL4* DNA-binding domain fusion vector (22, 23). Human *CDC34* in the KS^+ plasmid was cut with *Not*I followed by *Sma*I at the codon encoding the first methionine and inserted in frame into the vector. The ability of the pPC97-*hCDC34* construct to encode the functional Cdc34 protein was tested in yeast by its ability to complement a temperature sensitive $cdc34^{ts}$ mutant yeast strain, SJ1098-3d (Mat **a**, *cdc34-2*, *leu2-3*, *ura3*, *trp1*- B. Byers, University of Washington, Seattle) (Fig.1). After transformation, cells were spread onto plates containing leucine-deficient medium and kept at 23°C for 24h, after which they were shifted to either 30°C or 37°C and incubated for 3 days. The pPC97-*hCDC34* construct was able to completely suppress the $cdc34^{ts}$ mutation allowing growth at 37°C (Fig.1). Part of the DB-*CDC34* construct has also been verified by sequence analysis using an internal oligo 340 bp downstream of the 5'end of *CDC34*. We have also verified that the *GAL4*-Cdc34 fusion protein is expressed in the host two-hybrid strain (MV103) by Western blot using a monoclonal Cdc34 antiserum (Transduction Lab) (Fig.2). This protein has no autonomous effect on His3, Ura3 and LacZ reporters, due for example to the presence of cryptic transcriptional activation sequences. We also determined the minimal concentration of 3-amino triazole (3AT; 10-25 mM) to prevent growth of the pPC97-*hCDC34* bait.

Activation domain-cDNA library: A human T-lymphocyte cDNA fusion library in the activation domain vector pPC86 (Trp+) was kindly provided by J. La baer (MGH Cancer Center). The cDNAs were cloned into the *EcoRI* (5') and *SpeI* (3') sites. This library has approximately $2X10^6$ clones and the average insert size is 1kb. *hCDC34* is highly expressed in T-lymphocytes and the library appears to have a broad range of cDNAs and has been successfully used in two-hybrid screening (Vidal M. et al unpublished). This library has not been amplified in liquid culture at anytime. However, we have amplified this library once by electroporation using electrocompetent *E. coli*, JS4 cells (BioRad) followed by replica plating onto LB+Ampicillin plates. The DNA was prepared using a Plasmid Maxi kit from Qiagen.

Selection of *CDC34* interacting genes: A key to the success of a two-hybrid screen is sufficient transformation efficiency to allow screening of at least one million clones. We have optimized the transformation protocol using the lithium acetate method (Schiestl and Giets, 1989, Durfee et al, 1993) which is highly efficient and reproducible. We have developed a modified procedure using yeast total RNA and denatured salmon sperm DNA together as carriers, which achieved a transformation efficiency of 300,000 colonies per microgram

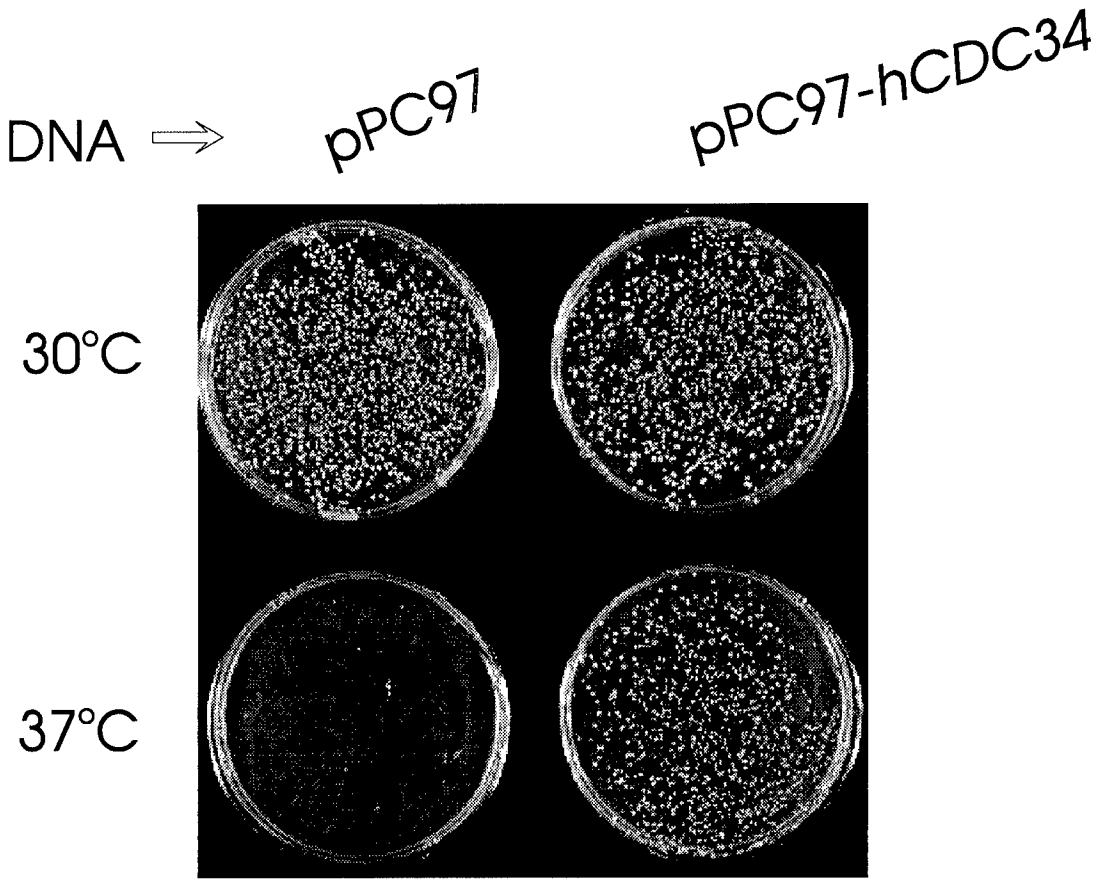

Fig.1. PC97-*hCDC34* bait encodes the functional Cdc34 protein. This was tested in yeast by its ability to complement a temperature sensitive *cdc34*^{ts} mutant yeast strain, SJ1098-3d (Mat **a**, *cdc34-2*, *leu2-3*, *ura3*, *trp1*). After transformation with the plasmid DNA, pPC97 or the pPC97-*hCDC34*, cells were spread onto plates containing leucine-deficient medium and kept at 23°C for 24h, after which they were shifted to either 30°C or 37°C and incubated for 3 days. Unlike the control plasmid (pPC97), the pPC97-*hCDC34* construct was able to completely suppress the *cdc34*^{ts} mutation, allowing growth at 37°C.

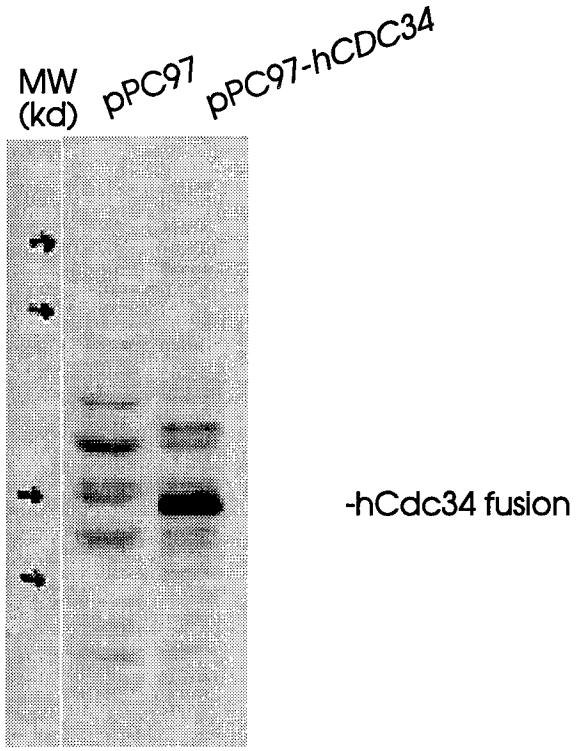

Fig. 2. Western blot analysis of the *GAL4*-Cdc34 fusion protein, expressed in the host two-hybrid strain (MV103). Protein extracts made from overnight cultures of yeast bearing the control plasmid (pPC97) or the bait (pPC-97-hCDC34) were electrophoresed on a 12% SDS-PAGE gel and transferred onto a Immobilon-P membrane (Millipore) using BIO-RAD Mini-Trans blot apparatus. The membrane was then probed using a monoclonal anti hCdc34 antiserum (Transduction Lab). The left hand panel shows the molecular weight marker (in kd).

plasmid DNA, a substantial improvement over previous protocols (Pati, D. and Plon, S.E., unpublished). With this transformation protocol, we have sequentially transformed the bait (Leu+) and the library plasmid (Trp+) into the yeast host strain MV103. The transformants containing the bait and library plasmids were selected on media lacking leucine and tryptophan. Three separate pools of library DNA were used to transform the MV103+pPC97-*CDC34* cells and 500,000 transformants from each pool were obtained.

The screen was carried out by first selecting for growth of Cdc34 bait-library co-transformants on Sc-His-Leu-Trp+25mM 3AT. Subsequently additional reporter genes, *URA3* and *LacZ*, were selected for in the 3AT positive clones. The expression of the *URA3* gene was both selected for on media lacking uracil as well as counterselected against on media containing uracil and 0.1% 5-fluoroorotic acid (5FAO). Induction of the *LacZ* gene was assayed qualitatively in the presence of X-Gal for blue colonies. The phenotypes were then scored (Table 1).

Table1: Summary of the *hCDC34* two-hybrid screen.

Total number of cDNA clones screened: 1.5 million
Clones positive for secondary selection (His/3AT): 643
Clones positive for tertiary selection (His/3AT+, Ura+, FOA-, Xgal+): 30
Clones sequenced: 18 (14 known genes, 4 novel clones)

In the screen out of the 1.5 million transformants 30 clones were found positive for all the reporters (His/3AT+, Ura+, FOA-, Xgal+) whereas 60 others were partially positive (3AT+, Ura+, FOA +/-, X-Gal +/-). Out of these 30 clones, 18 clones have been sequenced; seven of them are known cell cycle regulators and four are novel clones. Surprisingly, four of these 18 positive clones represent genes which have been previously identified in the regulation of meiosis and spermatogenesis, one also has a distinct role in DNA double strand break repair. These genes are also known to be expressed in lymphocytes, presumably due to the requirement for recombination of immunoglobulin and T cell receptor genes in lymphocytes. Thus, it appears from our studies that human Cdc34 may have a novel role in meiosis, recombination, and response to DNA damage in higher organisms by specifically targeting these regulators for ubiquitination. Recently, several genes involved in DNA repair have been indicated in breast cancer development (26, 27). Further studies are currently in progress to characterize the Cdc34-mediated regulation of these interactors and their physiological role in the cell cycle progression and development of malignancy. In this regard, we have recently procured a panel of breast cancer cell lines, including MCF10, MCF7, MDA-MB-157, MDA-MB-231, MDA-MB-136, BT-20, HBL100, and SKBR-3 from ATCC. These cell lines were chosen due to previous analysis of the expression and structure of other human cell cycle genes including cyclins and BRCA1 (28, 29, 30). These cell lines are being maintained under similar conditions in DFCI media and isolation of DNA, RNA and proteins from these cells are underway. In addition, we intend to procure samples of primary breast tumors through the

Methodist Hospital Breast Tumor Tissue Bank (Houston, TX). This bank contains over two hundred primary breast tumors including a wide range of tumor types and stages of presentation. For many samples matched pairs of normal breast and tumor cells are available for mutational analysis. We will use these reagents to study the gene structure, and expression of the human Cdc34 and its interacting proteins using Southern, Northern and Western blot analysis.

Conclusion

Knowledge gained about mammalian Cdc34 function and its interacting partners will be instrumental in understanding how selective destabilization of proteins through ubiquitination regulates both the meiotic and mitotic cell division cycles and the role of Cdc34 in the development of breast cancer and oncogenesis in general. Eventually this knowledge can be used to develop therapies which modulate the stability of tumor suppressor and oncogene proteins.

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Appendix-I

Tasks Completed:

Technical objective #1: Identification of Cdc34 target proteins.

Task 1: Month 1-3: Cloning of *CDC34* cDNA (the bait) into DNA-binding domain vector pAS2 and determination of stable expression in yeast.

Task 2: Month 2-3: Excision of the library (the prey) encoding candidate interacting proteins fused to the activation domain from λACT.

Task 3: Month 4-6: Transformation of library plasmids into yeast host strains containing bait with sequential selection.

Task 4: Month 6-12: Determination and elimination of false positives.

Task 5: Month 10-16: Isolation and sequencing of the activation-domain fusion plasmids from true positives.

Tasks in Progress:

Task 6: Month 14-24: Independent biochemical confirmation of authentic two-hybrid interactions.

Technical objective #2: Role of Cdc34 and its interacting proteins in carcinogenesis.

Task 7: Month 18-28: Study of the structure of human *CDC34* and its novel partner proteins in malignant mammary cells.

Task 8: Month 18-28: Study of the expression of human *CDC34* and its novel partner proteins in malignant mammary cells.

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7/19/2000

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